

FvVE1* regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in *Fusarium verticillioides

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Summary

The velvet gene, *veA*, co-ordinates asexual and sexual development in the homothallic fungal species *Aspergillus nidulans*. Studies in *Aspergillus parasiticus* and *Aspergillus fumigatus* demonstrated that *veA* also regulates morphological differentiation in these species. Whether *veA* has the same role in morphogenesis in other fungal genera has not been investigated. In this work, we studied the role of the *veA* homologue, *FvVE1*, in the heterothallic fungus *Fusarium verticillioides*. Deletion of *FvVE1* suppressed aerial hyphal growth and reduced colony surface hydrophobicity on solid media. In submerged cultures, *FvVE1* deletion caused alterations in hyphal polarity, marked activation of conidiation and yeast-like growth. The latter was promoted by shaking to increase aeration of cultures. In addition, *FvVE1* deletion markedly increased the ratio of macroconidia to microconidia. Supplementation of osmotic stabilizers restored the wild-type phenotype to deletion mutants, suggesting phenotypic alterations caused by *FvVE1* deletion are related to cell wall defects. This is consistent with the hypersensitivity of *FvVE1* deletion mutants to SDS and with the significant reduction in the mannoprotein content of mutants compared with the wild-type strain. However, no dramatic cell wall alterations were observed when mutants were examined by transmission electron microscopy. Our data strongly suggest that *FvVE1* is important for cell wall integrity, cell surface hydrophobicity, hyphal polarity and conidiation pattern.

Introduction

Fusarium verticillioides (synonym: *Fusarium moniliforme*, teleomorph: *Gibberella moniliformis*; synonym *Gibberella fujikuroi* mating population A) is a filamentous fungus that causes stalk and ear rot of corn (Leslie *et al.*, 1990). In addition, *F. verticillioides* is the *Fusarium* species most frequently isolated from infected and apparently healthy corn seed (Macdonald and Chapman, 1997; Desjardins *et al.*, 2000).

Fusarium verticillioides can infect maize through root, shoot and seed in various developmental stages of healthy plants. Conidia are the primary source of inoculum (Fandohan *et al.*, 2003). Most *Fusarium* species have two distinct conidial forms, the macroconidium and microconidium. In *F. verticillioides*, single-celled microconidia (7–10 µm × 2.5–3.2 µm) are the most abundant asexual spore form, while the larger and septated macroconidia (31–58 µm × 2.7–3.6 µm) are typically much less abundant (Nelson *et al.*, 1983; Sutton *et al.*, 1998). *F. verticillioides* has two mating types controlled by a locus with two alternate idiomorphs (*MAT1-1* and *MAT1-2*) containing a conserved alpha box domain and a high-mobility-group (HMG) box domain (Kerényi *et al.*, 1999; Steenkamp *et al.*, 2000; Yun *et al.*, 2000). Both mating types are widespread in natural populations (Leslie, 1995). To date, the only genes reported to affect morphogenesis in this important plant pathogen are the *FPH1* gene, the hydrophobin genes, *HYD1* and *HYD2*, and the cyclin-C-encoding *FCC1* gene (Shim and Woloshuk, 2001; Fuchs *et al.*, 2004; Glenn, 2006 respectively). The *fph1* mutant presented an aconidial phenotype, while the hydrophobin mutants, *hyd1* and *hyd2*, produced microconidia in false heads rather than in chains like the wild type (Fuchs *et al.*, 2004; Glenn, 2006). Finally the *fcc1* mutant exhibited a pH-dependent reduction in conidial formation (Shim and Woloshuk, 2001).

Most knowledge on mechanisms of morphogenesis in filamentous fungi has been obtained using the homothallic fungus *Aspergillus nidulans* as a model system. From the *A. nidulans* studies, many developmental genes have been identified and characterized (Adams *et al.*, 1998; Calvo *et al.*, 2002). Among them, the velvet gene, *veA*, is an important regulator of asexual and sexual develop-

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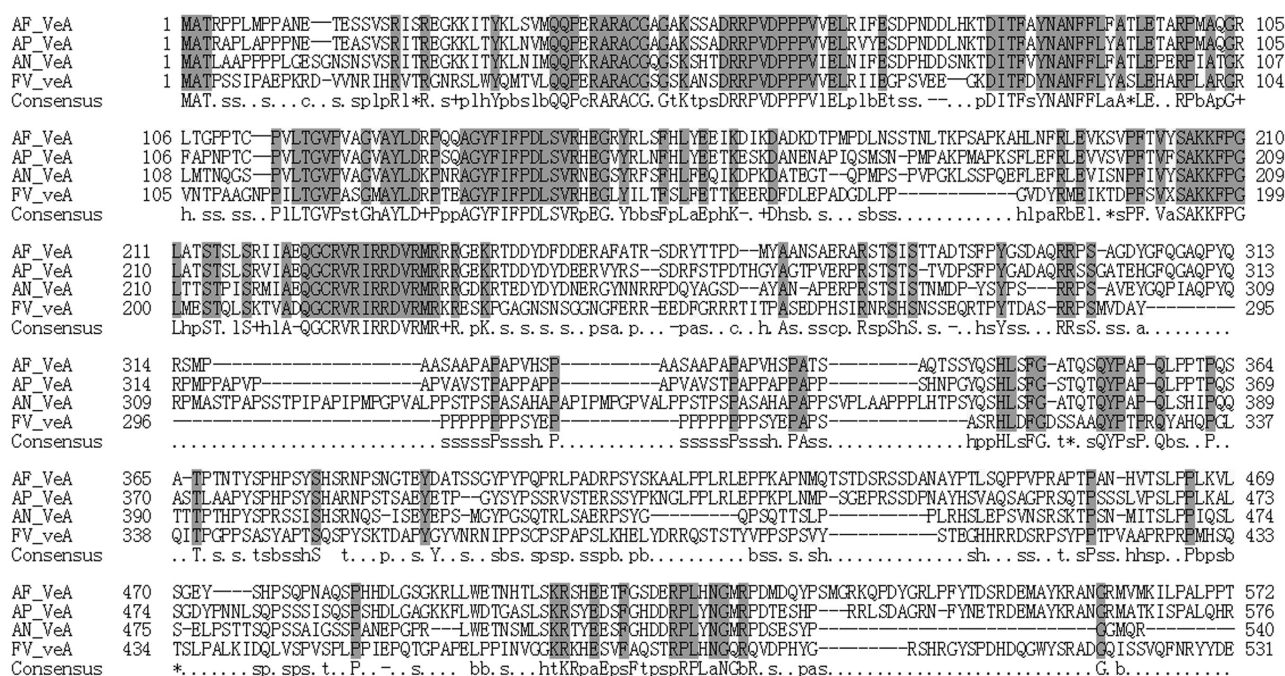


Fig. 1. Multiple sequence alignment of *FvVE1* with other characterized *VeA* homologous sequences. The alignment was generated with ClustalX software (Thompson *et al.*, 1997) and coloured with Chroma (Goodstadt and Ponting, 2001). The conserved amino acids are shaded. Polar residues are highlighted in yellow. The consensus sequence is reported below the alignment. Negative symbol indicates negative residues, positive symbol indicates positive residues, 1, aliphatic; a, aromatic; c, charged; t, tiny; s, small; b, big; p, polar; h, hydrophobic (all below the alignment). AN_VeA, AF_VeA, AP_VeA and FV_VeA are abbreviations of *VeA* homologues in *Aspergillus nidulans* (Accession number: U95045), *Aspergillus fumigatus* (Accession number: XM_747526), *Aspergillus parasiticus* (Accession number: AY445513) and *Fusarium verticillioides* (Accession number: DQ274059).

ment (Käfer, 1965; Yager, 1992; Calvo *et al.*, 2002; Kim *et al.*, 2002; Kato *et al.*, 2003). Although the *veA* gene product does not exhibit significant homology to known proteins, its role in morphogenesis is clear. Mutations in *veA* abnormally activated conidiation (Käfer, 1965; Kim *et al.*, 2002) and abolished sexual development (Kim *et al.*, 2002; Kato *et al.*, 2003), while overexpression of *veA* has opposite effects (Kim *et al.*, 2002). The *veA* homologue was also found to control conidiation and sclerotial production in *Aspergillus parasiticus* (Calvo *et al.*, 2004), and conidiation in a nitrate-dependent manner in *Aspergillus fumigatus* (Krappmann *et al.*, 2005). The role of *veA* homologues in fungal genera other than *Aspergillus* has not been reported.

In this study, we explored the role of the *veA* homologue, *FvVE1*, in growth and morphological development in *F. verticillioides* as a model system for heterothallic fungi. Specifically, we examined the phenotypic characteristics of *FvVE1* deletion strains ($\Delta Fvve1$ strains), including growth and conidiation in the two mating types. The results indicate that *FvVE1* affects cell wall integrity, hyphal surface hydrophobicity, hyphal growth and the ratio of microconidia versus macroconidia in *F. verticillioides*.

Results

Characterization of the *F. verticillioides* *FvVE1* sequence

This is the first report of a *veA* homologue in a genus other than *Aspergillus*. The deduced amino acid sequence of the *F. verticillioides* *FvVE1* gene (GenBank Accession number DQ274059) exhibited 33% identity and 61.6% similarity with the *A. nidulans* *VeA* protein sequence (Accession number U95045), 36.4% identity and 64.6% similarity with the *A. parasiticus* sequence (Accession number AY445513), and 36.5% identity and 65.4% similarity to the *A. fumigatus* sequence (Accession number AAHF01000004). The N-terminal region was more highly conserved than other regions of the protein (Fig. 1). The highly conserved N-terminal region does not exhibit significant homology with any known domains. The C-terminal region is variable even among *Aspergilli* *veA*. The *FvVE1* and the *A. nidulans* *veA* genes are dissimilar enough that complementation of the *A. nidulans* *veA* deletion mutant (described in Kim *et al.*, 2002; Kato *et al.*, 2003) with the *FvVE1* wild-type allele did not rescue the *A. nidulans* wild-type phenotype, resulting in small colonies with abundant aerial mycelium (Fig. S1).

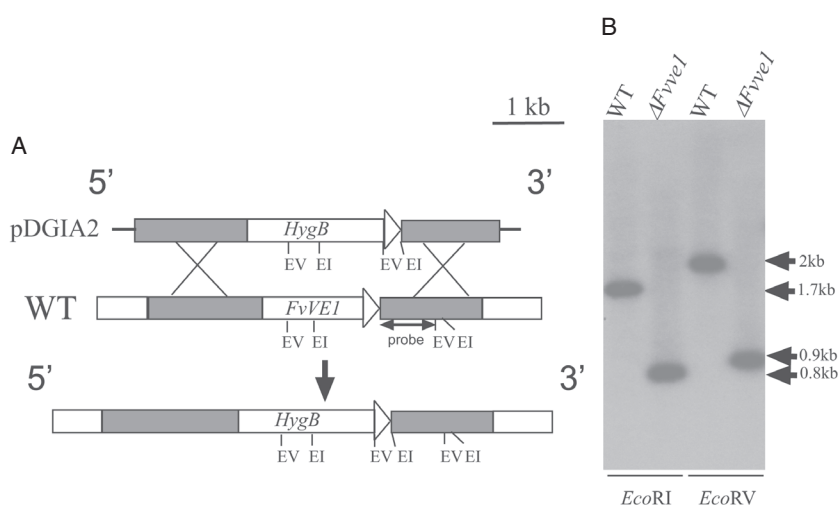


Fig. 2. Generation of *F. verticillioides* *FvVE1* deletion mutants. A. Schematic diagram of the *FvVE1* deletion vector pDGIA2.

B. Southern hybridization analysis of hygromycin B resistant strains following transformation of wild-type MAT 1-1 strain M-3125 with pDGIA2. Southern blots for strains recovered following transformation of MAT1-2 strain M-3120 were identical.

Deletion of FvVE1 suppressed formation of aerial hyphae and reduced hydrophobicity of the hyphal surface

Deletion of *FvVE1* was confirmed by polymerase chain reaction (PCR) (data not shown) and Southern analysis (Fig. 2) as specified in the Experimental procedure section. *FvVE1* deletion dramatically affected colony morphology in an inoculation method-dependent manner. When conidia were streaked on the surface of V8 medium, wild-type strains formed fluffy colonies with abundant aerial hyphae and microconidial chains. In contrast, $\Delta Fvve1$ mutants formed dense, slimy colonies without aerial hyphae or microconidial chains (Fig. 3A). In the wild-type cultures, colonies continued to grow until they formed confluent growth. In contrast, in mutant cultures, colonies remained discrete and did not form confluent growth (Fig. 3A). When conidia were point-inoculated on the same medium, $\Delta Fvve1$ mutants formed radial colonies with a slightly lower growth rate than that of wild-type strains. Therefore, the mutants did not lose the ability to form radial colonies. Interestingly, while the wild-type colonies formed from point inoculations had abundant aerial hyphae giving the colony a white appearance, the $\Delta Fvve1$ mutant colonies had dramatically reduced aerial hyphae and were light brown (Fig. 3B).

The hydrophobic property on the cell surface is a distinguishable feature of aerial hyphae and has been described as contributing to their formation in many fungal species (Kershaw and Talbot, 1998; Wösten *et al.*, 1999). Reduction of aerial hyphae in $\Delta Fvve1$ mutants suggests a reduction of hydrophobicity of the cell surface. To compare the hydrophobicity of the colony surface, 30 μ l of water was placed on the surface of mycelia grown on the solid medium YPGA (to provide a clear image background). On colonies of wild-type strains, the water

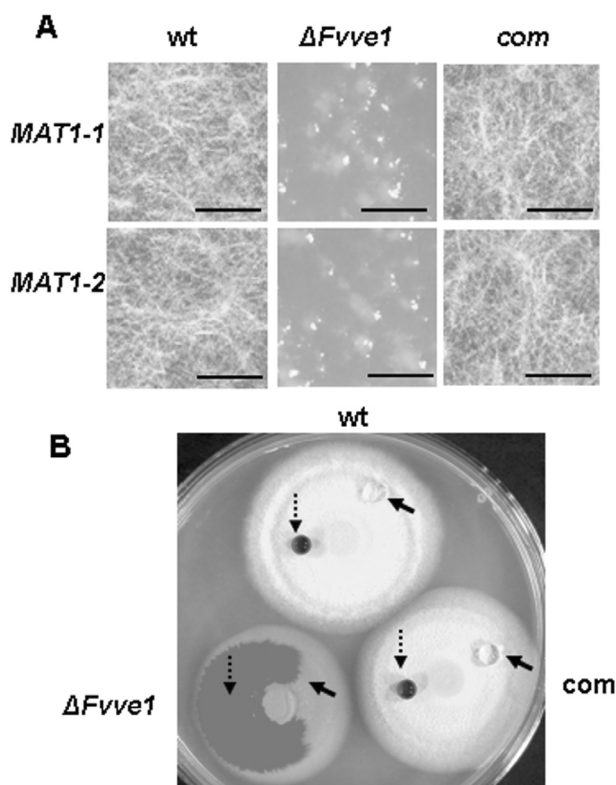


Fig. 3. Colony morphology of wild-type strains (wt, M-3120), $\Delta Fvve1$ mutants (M31206) and complemented strains (com, M31206C5) of *F. verticillioides*.

A. Conidia were inoculated by streaking plates on V8 agar medium and incubated for 5 days. Images were captured with a Spot Insight Color camera on a Leica MZ75 dissecting microscope with a 2.5 \times objective. Bar, 1 mm.

B. Conidia were spotted on V8 agar plates with toothpicks and incubated for 3 days. Thirty microlitres of water or 1% acid fuchsin droplets (red spots) were loaded on colony surfaces. The loading locations were indicated by arrows.

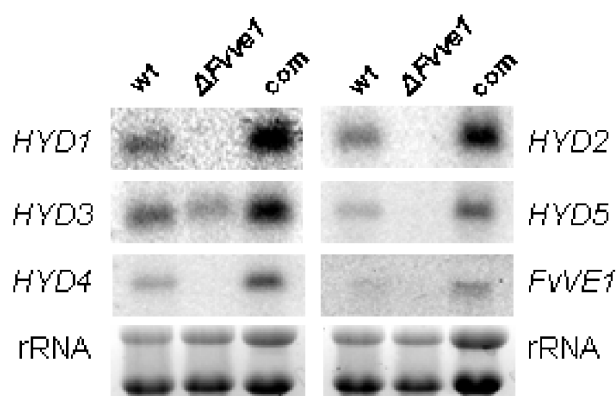


Fig. 4. Northern hybridization of total RNA from the of wild-type strain (wt, M-3120), $\Delta Fvve1$ mutant (M31206) and complemented strain (com, M31206C5) of *F. verticillioides* using ^{32}P -labelled *HYG1-HYD5* and *FvVE1* gene probes. The ethidium bromide-stained rRNAs are shown to indicate RNA loading.

formed a spherical droplet on the surface of the mycelium without extending or being absorbed for at least 30 min. In contrast, on $\Delta Fvve1$ mutant colonies, the water was absorbed into the mycelium within 2 min. The absorption difference was easily visualized when 1% acid fuchsin water solution was placed on the colony surface (Fig. 3B). $\Delta Fvve1$ mutants that were transformed with a wild-type *FvVE1* allele (complementation strains) exhibited wild-type colony morphology under all conditions examined (Fig. 3). These experiments suggest that *FvVE1* is important for colony surface properties in *F. verticillioides*.

FvVE1 deletion altered the expression of hydrophobin genes

To test whether the loss of hydrophobicity in the *FvVE1* deletion strain was caused, at least in part, by an altered expression of hydrophobin genes, *HYD1*, *HYD2*, *HYD3*, *HYD4* and *HYD5* (Fuchs *et al.*, 2004), we performed Northern blot analyses for each *HYD* gene. Our results revealed that deletion of *FvVE1* caused a severe reduction of *HYD1-HYD5* transcripts. *HYD3* gene expression was strongly reduced and the expression of *HYD1*, *HYD2*, *HYD4* and *HYD5* was completely blocked in the mutant strain under conditions that allowed the expression of these genes in the wild-type and complementation strains (Fig. 4). Under the same experimental condition, *FvVE1* transcripts were detected in the wild-type and complementation strains but were not detected in the mutant strain as expected (Fig. 4).

FvVE1 deletion reduced ratio of microconidia : macroconidia

Wild-type *F. verticillioides* generally produces many more microconidia than macroconidia (Nelson *et al.*, 1983; Sutton *et al.*, 1998; Fig. 5). However, $\Delta Fvve1$ mutants produce more macroconidia than microconidia (Fig. 5). This alteration in conidiation was observed in both mating types grown on V8 agar (Fig. 5) and on YGT, carnation leaf agar and YPGA media (data not shown) media as well as in dark or continuous light conditions. These results indicate that the alteration of conidial pattern caused by *FvVE1* deletion was independent of medium and light

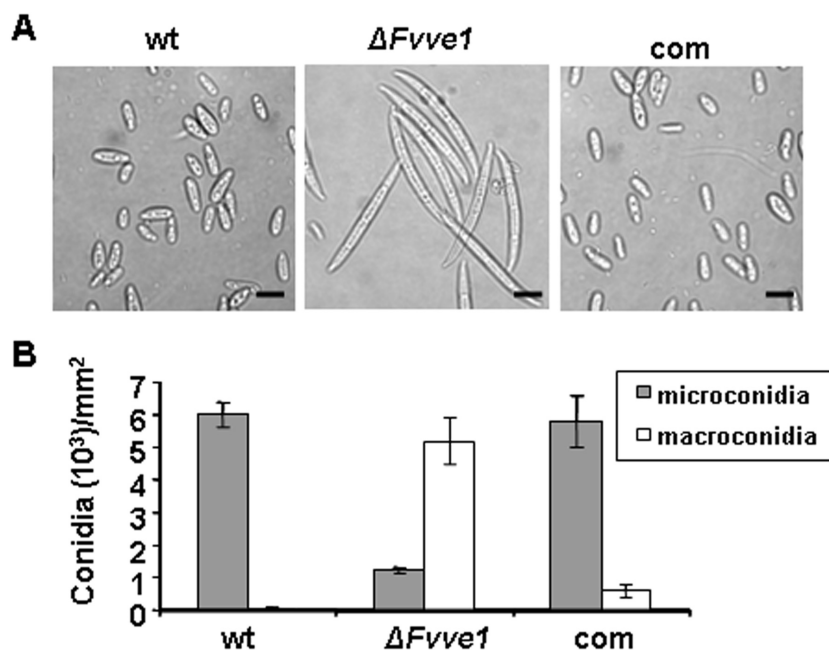


Fig. 5. Alteration in microconidium and macroconidium production in $\Delta Fvve1$ mutants. **A.** Micrograph of conidia produced by wild-type strain M-3120 (wt), $\Delta Fvve1$ mutant strain M31206 and complementation strain (com, M31206C5) are shown. Images were captured with NikonZ995 camera attached to Nikon Eclipse E400 microscope with a 40 \times objective. Bar, 10 μm . **B.** Quantification of conidia. Values are the mean number of conidia per mm recovered from 12.6 mm² plugs taken from triplicate V8 agar cultures. Error bars indicate standard deviation.

conditions. Complementation strains exhibited wild-type conidiation (Fig. 5). The morphology of the different conidia produced was further confirmed by examining nuclei number and septa using the nucleus-specific 4',6-diamidino-2-phenylindole (DAPI) and cell wall-specific calcofluor staining procedures (Fig. S2). These results indicate that *FvVE1* is required for production of the normal ratio of microconidia/macroconidia in *F. verticillioides*.

Deletion of *FvVE1* resulted in altered germination

Germination of conidia of wild-type and $\Delta Fvve1$ mutant strains differed from in/on all media examined (at the inoculation origin on solid media – V8, YGT and YPGA data not shown – as well as in liquid media). Independently of the type of inoculation method, germination of conidia of wild-type strains of *F. verticillioides* always gave rise to filamentous growth. In static liquid YPG medium, wild-type conidia germinated to form unbranched germ tubes within 12 h of inoculation (Fig. 6A). After 24–48 h of incubation, germ tubes elongated towards the liquid–air interface where they produced conidia. Under the same conditions, $\Delta Fvve1$ conidia exhibited three types of germination, all of which differed from the wild type. First, $\Delta Fvve1$ conidia germinated to form short, highly branched germ tubes (Figs 6A and 8A). We will hereafter refer to this growth phenotype as altered polarized growth. In some cases, these short, highly branched germ tubes produced conidia. Second, $\Delta Fvve1$ conidia germinated to form short unbranched germ tubes that subsequently produced another conidium (Figs 6A and 8A). Some authors define this latter type of growth as microcycle conidiation (Maheshwari, 1991; Griffin, 1994; Khurana *et al.*, 1996; Cousin *et al.*, 2006). Third, some $\Delta Fvve1$ conidia did not germinate to form germ tubes but instead produced a second conidium directly from the mother conidium in a manner resembling the budding growth of the yeast *Saccharomyces cerevisiae* (Fig. 6A), and was called yeast-like growth in this study. DAPI and calcofluor staining of nuclei and cell walls revealed that $\Delta Fvve1$ conidia formed from all three types of growth were mononucleate and did not have septa. However, they were in general notably larger than the microconidia produced by wild-type and complementation strains (an example is shown in Fig. S2).

In contrast to wild-type strains, $\Delta Fvve1$ mutants did not display defined growth orientation, most likely due to hyper-branching, and their conidiation did not require the contact of hyphae or conidia with the liquid–air interface. As a result, total conidial production of $\Delta Fvve1$ mutants was 35-fold greater than that of wild type in static liquid YPG cultures after 48 h incubation (Fig. 6B).

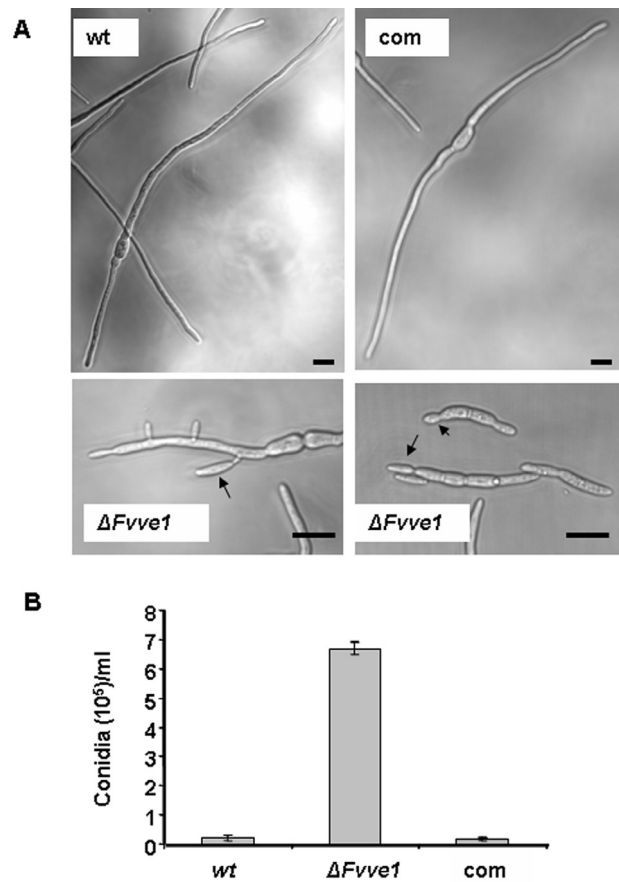


Fig. 6. Phenotypic characteristics of $\Delta Fvve1$ mutants in static liquid culture.

A. Images were obtained from a 12-h-old culture using a Zeiss LSM 5 pascal confocal laser scanning microscope. Conidia produced in submerged culture are labelled with arrows. Wild-type strains (wt, M-3120), $\Delta Fvve1$ mutants (M31206) and complemented strains (com, M31206C5) are shown. Bar, 30 μ m.

B. Quantitative comparison of conidium production in stationary submerged culture after 48 h incubation. Values are the mean number of conidia per millilitre in three independent replicate Petri dishes. Standard deviations from three replicate dishes are shown.

The germination phenotypes of $\Delta Fvve1$ mutants described above were enhanced by shaking, which improves aeration. When grown in liquid YPG medium with shaking, wild-type strains produced more microconidia than in static liquid culture, but produced abundant hyphae as well (data not shown). In contrast, $\Delta Fvve1$ mutants proliferated mainly by forming hyper-branched hyphae, microcycle conidiation and yeast-like growth. Although the type of growth differed, we noticed that the amount of biomass produced by wild type and mutant strains after 24 h incubation was similar (data not shown). The phenotypic characteristics of $\Delta Fvve1$ mutants in liquid culture were observed in both the *MAT1-1* and *MAT1-2* genetic backgrounds. Complementation strains exhibited wild-type growth in liquid culture (Fig. 6). These results indicate that *FvVE1* is required for

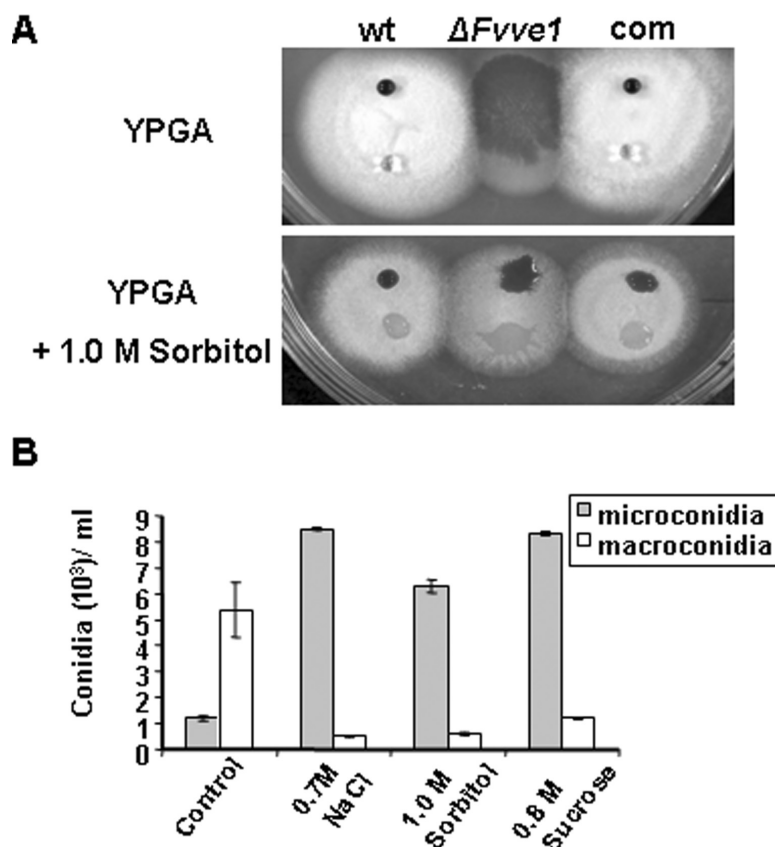


Fig. 7. Restoration of the wild-type phenotype in $\Delta Fvve1$ mutants grown on V8 agar medium amended with osmotic stabilizers. Wild-type strains (wt, M-3120), $\Delta Fvve1$ mutants (M31206) and complemented strains (com, M31206C5) are shown.

A. Conidia were spotted on YPGA plates (with or without 1 M sorbitol) with a toothpick and incubated for 3 days. Thirty microlitres of water or 1% acid fuchsin droplets (red spots) were applied to colony surfaces. The loading locations are indicated by arrows. Images were captured with a Spot Insight Color camera on a Leica MZ75 dissecting microscope with a $2.5\times$ objective.

B. Quantification of conidia in the presence and absence of osmotic stabilizers. V8 agar medium supplemented with various osmotic stabilizers was inoculated by streaking the agar surface with conidia. The resulting cultures were incubated for 5 days. Values are the mean number of conidia per millilitre recovered from 12.6 mm^2 plugs taken from three replicate V8 agar cultures. Error bars indicate standard deviation.

normal filamentous growth and conidiation in *F. verticillioides*.

FvVE1 deletion affected sexual development

When a wild-type culture was fertilized with wild-type conidia abundant perithecia were produced over the entire surface of the carrot agar medium after 7 days. In contrast, when a wild-type strain was fertilized with $\Delta Fvve1$ conidia, production of perithecia was rare (Fig. S3). No perithecia were observed on $\Delta Fvve1$ fertilized by either wild-type conidia or $\Delta Fvve1$ conidia (Fig. S3). All the complementation strains still showed the infertility phenotype (data not shown).

Osmotic stabilizers restored aerial hyphae formation, hydrophobicity, normal ratio of microconidia : macroconidia and normal hyphal polarity to $\Delta Fvve1$ mutants

The reduced hydrophobicity of the cell surface in *FvVE1* deletion mutants suggest an alteration in cell wall composition, and increased branching frequency can be a consequence of cell wall defects in *Aspergillus* species (Bormann *et al.*, 1999; Muller *et al.*, 2002). In addition,

increased conidial size is likely caused by the reduced resistance of cell walls to turgor pressure. To test whether the phenotypic alterations exhibited by $\Delta Fvve1$ mutants are related to cell wall defects, we compared the hyphal growth and conidiation of the wild-type progenitor, the mutant, and complemented strains on V8 agar medium in the presence and absence of the osmotic stabilizers, 0.7 M NaCl, 1.0 M sorbitol, or 0.8 M sucrose. Osmotic stabilizers, which are able to reduce cellular turgor pressure, can be used to restore wild-type growth in mutants of *S. cerevisiae* (Torres *et al.*, 1991; Levin and Bartlett-Heubusch, 1992) and *A. nidulans* (Borgia *et al.*, 1996) with cell wall defects. $\Delta Fvve1$ colonies were markedly more similar to those of wild-type and complementation strains in the presence of the osmotic stabilizers (Fig. 7A). The radial growth rate of $\Delta Fvve1$, wild-type and complementation strains did not differ significantly in the presence of the stabilizers. In addition, $\Delta Fvve1$ mutants exhibited a dramatic increase in production of aerial hyphae and in mycelial hydrophobicity in the presence of the osmotic stabilizers. This result suggests that osmotic stabilizers alter the properties of cell surface in addition to balancing turgor pressure. Moreover, the osmotic stabilizers restored the wild-type ratio of microconidia to macroconidia in the mutants

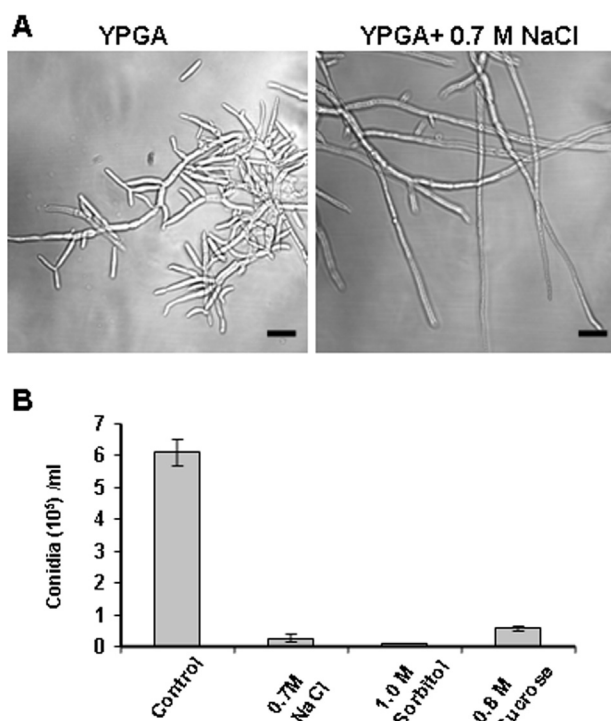


Fig. 8. Restoration of the wild-type phenotype in $\Delta Fvve1$ mutant in static submerged culture by osmotic stabilizers.

A. Micrographs of hyphal growth. Conidia of $\Delta Fvve1$ mutant strain M31206 were inoculated into YPG medium (with or without 0.7 M NaCl) and incubated for 24 h. Images were captured on a Zeiss LSM 5 pascal confocal laser scanning microscope with a 40 × objective. Bar, 30 μ m.

B. Quantification of conidia in the presence and absence of osmotic stabilizers after 48 h incubation. Values are the mean number of conidia per millilitre of YPG medium from three replicate cultures. Error bars indicate standard deviation.

on V8 agar medium (Fig. 7B), suggesting that the increased macroconidium production in $\Delta Fvve1$ mutants is likely caused by weakened cell wall.

The osmotic stabilizers also reduced the extensive branching and yeast-like growth exhibited by $\Delta Fvve1$ mutants in static liquid YPG medium. Like wild-type strains, $\Delta Fvve1$ mutants exhibited polarized hyphae in the presence of osmotic stabilizers (Fig. 8A; data for addition of 1.0 M sorbitol or 0.8 M sucrose not shown). In addition, conidiation of $\Delta Fvve1$ mutants in static liquid cultures with osmotic stabilizers (Fig. 8B) was reduced to wild-type

levels. Overall, osmotic stabilizers restored wild-type hyphal growth and conidiation in $\Delta Fvve1$ mutants.

FvVE1 regulates cell wall composition and integrity

The osmotic stabilizer-remediable phenotype of $\Delta Fvve1$ mutants suggests that *FvVE1* might regulate cell wall organization or synthesis. To address this, we tested sensitivity of the mutants to cell wall disrupting reagents including SDS and Calcofluor. When grown on YPD medium supplemented with 0.02–0.03% SDS, wild-type progenitor strains formed colonies that were morphologically the same as in the absence of SDS but that were reduced in radial growth. In contrast, $\Delta Fvve1$ mutants did not form colonies on SDS-containing YPD medium (data not shown). The sensitivity of $\Delta Fvve1$ mutants and wild-type strains to the chitin-binding chemical Calcofluor (200–300 μ g ml⁻¹) was similar on solid YPGA medium (data not shown) suggesting that the chitin level in $\Delta Fvve1$ mutants is similar to wild-type strains.

To test which cell wall components are altered in $\Delta Fvve1$ mutants, cell wall composition of both wild type and $\Delta Fvve1$ mutants was examined. Fungal cell wall composition was intensely studied in *S. cerevisiae*, in which cell wall is mainly composed with mannoprotein, β -1,3 glucan and chitin (Klis *et al.*, 2002). Mannoprotein is alkali soluble while β -1,3 glucan and chitin are mainly found in alkali insoluble fraction in yeast (Klis *et al.*, 2002). In this study we compared the same growth form, the cell wall composition of hyphae, harvested by filtration with Miracloth to eliminate conidia, between wild type and mutant. Two independent experiments revealed a small (8.6%) but statistically significant ($P < 0.0001$) reduction in mannoprotein content in $\Delta Fvve1$ mutant 31206 compared with wild-type strain M-3120, while glucan and chitin levels did not differ significantly in the mutant and wild-type strains (Table 1).

To test whether $\Delta Fvve1$ mutants have alteration in cell wall ultrastructure, ultra-thin sections of hyphae were observed by transmission electron microscopy. The thickness and appearance of cell walls of $\Delta Fvve1$ mutant 31206 was similar to that of wild-type strain M-3120 (Fig. 9), suggesting that slight alteration of cell wall composition caused by *FvVE1* deletion did not cause dramatic changes in cell wall structure.

Table 1. Composition of cell wall (μ g mg⁻¹ dry weight \pm standard deviation).

	Mannoprotein	Total glucan	Glucan in alkali soluble fraction	Chitin in alkali insoluble fraction
Wild type	271.5 \pm 4.5 A	48.4 \pm 3.4 A	34.9 \pm 1.5 A	27.7 \pm 2.2 A
$\Delta Fvve1$	250.0 \pm 4.1 B	48.8 \pm 3.6 A	34.5 \pm 1.1 A	29.4 \pm 2.6 A
<i>P</i> -value	< 0.0001	0.82	0.67	0.24

The means of the content of cell wall components from six replicates are displayed. The significance between wild type and mutant is labelled with A and B.

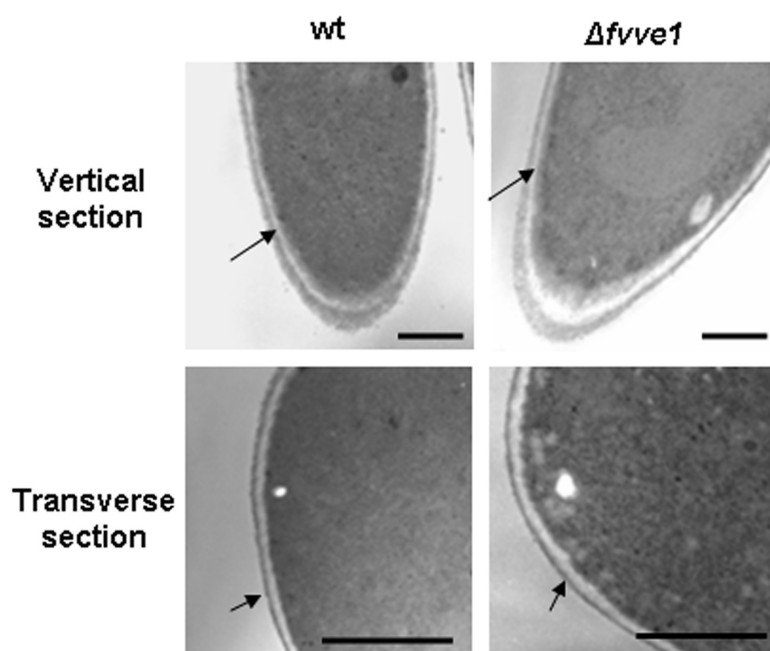


Fig. 9. Transmission electron microscopy of wild-type strain (M-3120) and $\Delta Fvve1$ mutant (M31206). Twelve-h-old mycelia grown in liquid YPG medium were fixed and stained with 2% uranyl acetate and 0.2% lead citrate. The cell wall is indicated by arrows. Bar, 10 μ m.

Discussion

FvVE1 regulates cell wall composition

The fungal cell wall confers rigidity and is important for viability and normal fungal morphology (Horiuchi *et al.*, 1999; Liu *et al.*, 2000; Muller *et al.*, 2002). Jeong *et al.* (2003) showed that deletion of the *veA* gene in *A. nidulans* resulted in a reduction of transcript levels of the mannoprotein-encoding gene *mnpA*. Because mannoproteins are integral components of the fungal cell wall, these results suggest a possible role for *veA* in regulating cell wall formation (the *mnpA* homologue has not been described in *F. verticillioides*). However, no phenotypic alteration of cell wall integrity was described in the *A. nidulans* *veA* deletion mutant (Jeong *et al.*, 2003). In the current study, we demonstrated that the *F. verticillioides* *FvVE1* homologue is required for normal cell wall composition and cell wall integrity. Remediation of mutant phenotypes with osmotic stabilizers, increased SDS sensitivity, and reduction in mannoproteins all indicate that *FvVE1* deletion mutants have cell wall defects. The osmotic stabilizer-remediable alterations, including changes in conidiation, hyphal polarity and aerial hyphae are striking. It is possible that cell wall proteins regulated by *FvVE1* could be important to the cell wall surface properties and normal morphology in *F. verticillioides*. These cell wall defect-related phenotypes exhibited by *Fvve1* mutants were not observed in the *veA* deletion mutant *A. nidulans*, thereby suggesting that the role of cell wall proteins regulated by *veA* homologous genes is species-dependent.

We also observed variations in the growth of *Fvve1* colonies depending on the method of inoculation. When conidia were spread over an agar medium surface, $\Delta Fvve1$ mutants formed small dense discrete colonies, while the wild-type strains form confluent colonies (Fig. 3A). However, when conidia were point-inoculated into the centre of an agar medium surface, mutant colonies grew radially at a rate just slightly slower than that in the wild type. One possible explanation for this type of growth could be a loss of recognition among mutant colonies.

In this study, we also demonstrated that *FvVE1* is required for hydrophobicity in *F. verticillioides*. Hydrophobicity is essential for fungal conidia and germlings to attach to the hydrophobic surfaces of plants (Braun and Howard, 1994). Although the work of Fuchs *et al.* (2004) on *HYD* genes showed that the individual deletion of the hydrophobin genes *HYD1–5* and the *HYD1* and *HYD2* double deletion did not alter the ability of *F. verticillioides* to cause maize seedling disease, these authors showed that the culture surface of *HYD* mutants (*HYD1–5* individually or *HYD1* and *HYD2* together) remained hydrophobic (Fuchs *et al.*, 2004). It is possible that overlapping *HYD* functions maintain hydrophobicity in these mutants or that other proteins also contribute to hydrophobicity. To elucidate the genetic regulation of *FvVE1* on hydrophobicity we analysed the effect of *FvVE1* deletion on *HYD* gene expression (Fig. 4). We found that *FvVE1* is required for normal expression of the five *HYD* genes, which could contribute to the lack of hydrophobicity in the mutant colonies. In addition, the decrease in aerial mycelium could also be associated with this phenotype.

FvVE1 regulated the balance between filamentous growth and conidiation

Many studies suggest that filamentous growth and conidiation are controlled by antagonistic mechanisms in fungi. For example, deletion of *fadA* or *pkaA* genes in *A. nidulans* and *gna-3* or *pkac-1* genes in *Neurospora crassa* reduces filamentous growth and causes premature conidiation (Yu *et al.*, 1996; Kays *et al.*, 2000; Shimizu and Keller, 2001; Banno *et al.*, 2005). Overexpression of genes such as *flbA* or *brlA* (Lee and Adams, 1994; Marhoul and Adams, 1995) also abnormally increases conidiation and reduces filamentous growth in *A. nidulans*. On the other hand, mutations in the genes such as *acoD* or *brlA* abolish conidiation and cause excessive vegetative growth, known as the fluffy phenotype, in *A. nidulans* (Boylan *et al.*, 1987; Adams *et al.*, 1992). Although great progress has been made in understanding the genetic regulation of conidiogenesis (Adams *et al.*, 1998; Calvo *et al.*, 2002), most of the research has been carried out using *A. nidulans*. It is in this model fungus, and later in *A. parasiticus* and *A. fumigatus* where *veA* homologues were described to regulate conidiation (Kim *et al.*, 2002; Calvo *et al.*, 2004; Krappmann *et al.*, 2005). Knowledge of the sporulation process in other fungal genera, including *Fusarium* species, is very limited. The economically important maize pathogen *F. verticillioides* produces abundant air-borne conidia, which constitute its main source of inoculum for maize infection. Here, we found that deletion of the *F. verticillioides* *FvVE1*, results in a reduction of hyphal growth and increased conidiation, indicating that *FvVE1* may regulate the balance between vegetative growth and conidiogenesis in the fungus. The mutants were also impaired in sexual development; ascospore-bearing perithecia did not develop when the mutants were used as the female or were drastically reduced when used as male parent (data not shown). However, in contrast to all the morphological phenotypes described, sexual development was not restored in the complemented mutants. It is unclear why sexual development was not restored while all other phenotypes examined were. PCR analyses demonstrated that integration of the complementation vector was ectopic in all the complementation strains examined (data not shown). This could be an indication that restoration of sexual development requires the complementation vector to integrate at the *FvVE1* locus and/or the original wild-type structure at the locus be restored.

Aerial growth suppression accompanied by vigorous conidiation was observed in *veA1* mutant in *A. nidulans* (Käfer, 1965). An increase in conidiation was also observed in the *A. nidulans* deletion *veA* strain (Kim *et al.*, 2002; Kato *et al.*, 2003), suggesting that *veA* homologues could have certain similar regulatory roles in filamentous

growth and conidiation across fungal genera. However, the formation of conidia directly from mother conidia rather than from a conidiophore in the *F. verticillioides* mutants has not been observed in any *veA* deletion mutants in *Aspergillus* spp. (Kim *et al.*, 2002; Kato *et al.*, 2003; Calvo *et al.*, 2004). This suggests that the *veA*-mediated mechanisms controlling conidiation in *Aspergillus* and *Fusarium* are not identical. Recently we found a bipartite NLS in *A. nidulans* *VeA* protein sequence (Stinnett *et al.*, submitted) that is absent in the *FvVE1* deduced amino acid sequence. In addition, complementation of the *A. nidulans* *veA* deletion mutant with *FvVE1* not only did not rescue the wild-type phenotype but led to a different mutant phenotype (Fig. S1). Thus, there are multiple lines of evidence indicating that the regulatory mechanisms of *VeA*-like proteins are not identical in *Aspergillus* and *Fusarium*.

In addition, oxygen is generally required for conidiation in filamentous fungi (Adams *et al.*, 1998). Increased microcycle conidiation and yeast-like growth in $\Delta Fvve1$ mutants by shaking, which improves aeration of liquid cultures, suggests that these defects could be a consequence of the extreme activation of conidiation. Although yeast-like growth was reported previously in wild-type strains of *F. verticillioides* grown at 37°C (Wang *et al.*, 1998–1999), this is the first report of a gene deletion mutant in this fungus that exhibits yeast-like growth. Based on our results, we propose that *FvVE1* is important in conferring filamentous growth in *F. verticillioides*. To date, this is the first gene found in *Fusarium* species that controls filamentous growth.

FvVE1 is required for the balanced production of microconidia and macroconidia

Production of both microconidia and macroconidia is a common phenomenon in *Fusarium*, and the ratio between the two conidial forms is species-dependent. For example, *Fusarium graminearum* produces only macroconidia, *F. verticillioides* generally produces more microconidia than macroconidia, and *Fusarium oxysporum* and *Fusarium solani* tend to produce similar numbers of these types of conidia (Nelson *et al.*, 1983; Sutton *et al.*, 1998). In *F. oxysporum*, the *REN1* gene, which is similar to the *medA* in *A. nidulans* (Busby, 1996), is necessary for production of both microconidia and macroconidia but does not affect production of chlamydospores (Ohara and Tsuge, 2004). Also in *F. oxysporum*, the *FoSTUA* gene, homologue of *stuA* in *A. nidulans* (Wu and Miller, 1997), has opposite effects on production of macroconidia and chlamydospores but does not affect production of microconidia (Ohara and Tsuge, 2004). These reports suggest the existence of independent mechanisms controlling the formation of different types of spores in *F. oxysporum*.

However, the mechanism regulating the ratio of microconidia and macroconidia was not investigated in *F. oxysporum* or any other fungi. Here, we demonstrate that *FvVE1* plays a role in maintaining the wild-type ratio of conidial types formed by *F. verticillioides*. *Fvve1* mutants consistently produced a higher ratio of macroconidia to microconidia than the wild-type progenitor strains, but osmotic stabilizers could restore the wild-type ratio of conidia. This suggests that conidial production in the mutants was corrected with rescue of cell wall defects. It is possible that a reduction in cell wall integrity increases the size of conidia by altering turgor pressure. A relationship between the ratio of conidia types and cell wall integrity has not been reported in previous studies.

In conclusion, our results revealed an important role of *FvVE1* in cell wall integrity, cell surface hydrophobicity, normal hyphal polarity and conidiation in *F. verticillioides*. The fact that all vegetative growth and morphology phenotypes observed in *Fvve1* mutant were remediated by osmotic stabilizers suggests a connection between these cellular functions and *FvVE1*-dependent osmotic regulation. These findings should contribute to reduction of *F. verticillioides*-induced corn diseases and mycotoxin contamination by providing basic knowledge on the regulation of growth and development in this fungus. In addition, differences between deletion phenotypes observed here in *F. verticillioides* and previously in *Aspergillus* species indicate that *veA* homologues may regulate different cellular processes in different fungi.

Experimental procedures

Strains, media and growth condition

All *F. verticillioides* strains used in this study are listed in Table 2. Solid V8 agar medium (10% V8 juice, 0.1% CaCO₃ and 1.5% agar) and YPG liquid medium (0.3% yeast extract, 1% peptone and 2% glucose) were used for characterization of vegetative growth and asexual development. YGT (0.5% yeast extract, 2% glucose, 1.5% agar and 1 ml of trace element solution (Käfer, 1977) per litre of medium), carnation leaf agar and YPGA (0.3% yeast extract, 1% peptone, 2% glucose and 1.5% agar) were also used for this purpose as specified in each case. All cultures were grown at 28°C.

Identification of *F. verticillioides* *FvVE1*

The *F. verticillioides* *veA* homologue, *FvVE1*, was obtained by degenerate PCR followed by a screening of the previously described cosmid library (Proctor *et al.*, 1999). A 300 bp PCR product was amplified with degenerate primers 5'-TACAA(CT)GCCAA(CT)TT(CT)TTCCT-3' and 5'-G(AG)AACTT(CT)TTGGC(AG)CTG(AT)AG-3', which were designed based on nucleotide consensus sequences of *veA* genes from *Aspergillus* species, *N. crassa* and *Magnaporthe grisea* (data not shown). *F. verticillioides* strain M-3125

Table 2. *Fusarium verticillioides* strains used in this study.

Strain	Genotype	Source
M-3125	<i>MAT1-1</i>	Leslie (1991)
M-3120	<i>MAT1-2</i>	Leslie (1991)
M312501	<i>MAT1-1; ΔFvve1::HygB</i>	This study
M31206	<i>MAT1-2; ΔFvve1::HygB</i>	This study
M312501C1	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312501C2	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312501C3	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312501C4	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312501C5	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312501C6	<i>MAT1-1; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M312010	<i>MAT1-2; ΔFvve1::HygB</i>	This study
M312012	<i>MAT1-2; ΔFvve1::HygB</i>	This study
M312014	<i>MAT1-2; ΔFvve1::HygB</i>	This study
M312017	<i>MAT1-2; ΔFvve1::HygB</i>	This study
M31206C1	<i>MAT1-2; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M31206C3	<i>MAT1-2; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M31206C5	<i>MAT1-2; ΔFvve1::HygB, FvVE1::GenR</i>	This study
M31206C6	<i>MAT1-2; ΔFvve1::HygB, FvVE1::GenR</i>	This study

genomic DNA was used as template. The PCR cycling conditions were: 94°C for 2 min; then 40 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 1 min; and then 72°C for 5 min. The identity of the PCR product was confirmed by sequence analysis using a CEQ 8000 Genetic Analysis System (Beckman Coulter). The *F. verticillioides* cosmid library was screened by hybridization with the ³²P-labelled PCR product following standard colony-lift procedures (Sambrook *et al.*, 1989). Partial sequence analysis of a cosmid clone (pcosFvVE1F12B) obtained from the screen revealed that it contained a full-length *FvVE1* gene. The sequence information was used to construct a *FvVE1* deletion vector.

Generation of *FvVE1* deletion strains

The hygromycin B resistance gene (*HygB*) was used as a selective marker to replace *FvVE1* gene in *F. verticillioides*. First, *HygB* was amplified by PCR from pUCH2-8 (Turgeon *et al.*, 1987; Alexander *et al.*, 1998) to introduce NotI sites at both ends of the gene. The PCR primers were 5'-CGATGCGGCCGCACCGGCTGCACATGTCAAGG-3' and 5'-GCATGCGGCCGCACAGTTAAATTGCTAACGCAGTC-3' (NotI sites are underlined). The resulting PCR product was ligated into EcoRV-linearized pT7Blue-3 (Novagen, WI) to obtain pHYG-NotI. Fragments corresponding to the 1.4 kb 5'-flanking and the 1.3 kb 3'-flanking regions of the *FvVE1* coding region were obtained by PCR with Pfu polymerase (Stratagene, CA) and inserted into the pHYG-NotI plasmid. The 5'-flanking region was amplified with primers 5'-CCAACAATGGTACCCGAGACC-3' and 5'-TAATGAGGCGCATGCGAACGCGCTG-3' (underlined sequences are artificial KpnI and SphI restriction sites respectively) and ligated into pHYG-NotI, via the KpnI/SphI sites in the polylinker region, to yield pDGA1. The 3'-flanking region was amplified with primers 5'-ATGACCAAGCTTGGTACTCTCGAG-3' and 5'-GTTTAGACTGAGCTCAACACAAC-3' (underlined sequences indicate HindIII and SacI restriction sites respectively) and ligated into pDGA1, via the HindIII/

SacI sites in the polylinker region, to yield *FvVE1* deletion vector pDGI A2.

Wild-type *MAT1-1* strain M-3125 and *MAT1-2* strain M-3120 were used as transformation host strains to generate *FvVE1* deletion mutants. The *FvVE1* deletion strains were obtained by a double homologous recombination event, in which the *Hyg* gene replaced the *FvVE1* coding region (Fig. 2A). For transformation, 10^8 *F. verticillioides* conidia were inoculated into 100 ml of YPG liquid medium and incubated for 7–8 h at 28°C in a rotary shaker at 250 r.p.m. The resulting germings were digested with driselase (25 mg ml⁻¹, Kyowa Hakko, Japan) and lysing enzymes from *Trichoderma harzianum* (5 mg ml⁻¹, Sigma, MO) in 20 ml of 0.7 M NaCl for 1–2 h at 30°C with shaking (100 r.p.m.) to generate protoplasts. The transformation of protoplasts with plasmid DNA (pDGI A2) was performed as previously described (Lu *et al.*, 1994). Protoplast regeneration and mutant screening were conducted as described in Shim and Woloshuk (2001), using 150 µg hygromycin B (MP Biomedicals, CA) per millilitre of water agar overlay medium.

Genomic DNA was extracted from the transformants according to standard protocols (Sambrook *et al.*, 1989) and PCR and Southern blot analyses were used (as described in Sambrook *et al.*, 1989) to confirm deletion of *FvVE1* in the transformants. In the PCR analysis, primers 5'-CTT TGATCTGGAACCTGCCGATGGCGATCTTCCACCC-3' and 5'-GCATCCGCTCTGGAGTATGG-3', which amplify a 718 bp fragment from wild-type *FvVE1*, were used to determine whether *FvVE1* was deleted. Transformants that did not yield this fragment were further analysed by Southern blot (Fig. 2B). In the Southern analysis, the hybridization probe consisted of the 675 bp region of DNA down stream of the *FvVE1* stop codon (Fig. 2A). This probe was prepared by PCR amplification with primers 5'-GTTTAGACTGAG CTCAACACAAC-3' and 5'-ATGACCAAGCTTGGTACTC TCGAG-3' followed by EcoRV digestion and recovery of the 0.67 kb fragment (Fig. 2A). Genomic DNA (5 µg) digested with EcoRI or EcoRV was hybridized with ³²P dATP-labelled probe (Fig. 1B). The Southern blot analysis identified five *FvVE1* deletion mutants (M31206, M312010, M312012, M312014 and M312017) from the *MAT1-2* progenitor strain and one deletion mutant (M312501) from the *MAT1-1* progenitor strain.

Complementation of *FvVE1* deletion mutants

To generate a construct for complementing the *FvVE1* deletion mutants, a 4083 bp fragment, spanning from 1148 bp upstream of the wild-type *FvVE1* translation initiation codon to 1272 bp downstream of the *FvVE1* translation termination codon, was amplified by PCR using Pfu polymerase with the following primers, 5'-GCTTAGTCTAAGCTTAAGCTTAC-3' and GTTTAGACTGAGCTCAACACAAC-3' (HindIII/SacI artificial restriction sites are underlined respectively). This amplified fragment was digested with HindIII and SacI and inserted into HindIII/SacI-digested plasmid pGEN-NotI which contains a geneticin resistant gene *GenR* (Desjardins *et al.*, 2004). The resulting complementation vector, pDG3, was transformed into *FvVE1* deletion mutant strains M312006 (*MAT1-2*) and M312501 (*MAT1-1*) via the same transformation protocol used to generate the deletion mutants except

that the selection medium contained 150 µg geneticin per millilitre instead of hygromycin. Four (M31206C1, M31206C3, M31206C5 and M31206C6) from *MAT1-1* and six (from M312501C1 to M312501C6) geneticin resistant transformants were identified as complementation strains using the same PCR analysis used for the initial PCR screen of deletion mutants; in this analysis a 718 bp fragment of *FvVE1* was amplified from strains that were complemented and no band was amplified from strains that were not complemented.

Complementation of the *A. nidulans* ΔveA with the *FvVE1* gene

To obtain the transformation vector to complement the *A. nidulans* ΔveA mutation with the *FvVE1* gene, first, a 2507 bp fragment spanning from 765 bp upstream of the wild-type *FvVE1* translation initiation codon to 508 bp downstream of the *FvVE1* translation termination codon, was amplified by PCR using the FidelityTaq PCR Master Mix, 2 × (USB, OH), M-3120 genomic DNA as template and the following set of primers: 06310-04046Hind forward primer 5'-NNNAAGCTTGTAT GAATAAAATCT ATGGTCGTGTG-3' and 06311-04047Hind reverse primer 5'-NNNAAGCTTTCTCACATTGAGGG TGAGTTC-3' (artificial HindIII sites are underlined). The PCR product was digested with HindIII and ligated to the *pyroA*-containing plasmid pSM3 (Yu *et al.*, 1996), previously digested with HindIII and dephosphorylated. The ligation resulted in the vector pFvVE1SM, that was used to transform the *A. nidulans* RNKT3.3 strain (*biA1*; *pyroA4*; ΔveA). Fungal transformation essentially followed that of Miller *et al.* (1985), with the modification of embedding the protoplasts into top agar (0.75%) rather than spreading them with a glass rod on solid medium. Seven *A. nidulans* transformants, prototrophic for pyridoxine, were confirmed to contain the transformation vector pFvVE1SM. Expression of the *FvVE1* gene in *A. nidulans* was confirmed by reverse transcription PCR (data not shown).

Conidiation studies

For solid cultures, a 10 µl conidial suspension (approximately 10^8 conidia ml⁻¹) was streaked on V8 agar plates and incubated in either the dark or continuous light (25 microeinsteins m⁻² s⁻¹) for 5 days. To count conidia, a 12.5 mm² plug was punched from the resulting culture, homogenized in 200 µl of distilled water and then vortexed briefly. For liquid culture, 10 µl of conidial suspension (approximately 10^8 conidia ml⁻¹) was inoculated into 20 ml of YPG in Petri dish (Ø10 cm) and incubated without shaking or into 40 ml of YPG in 125 ml flask with shaking (200 r.p.m.). Conidia were counted under the light microscope with a Bright-Line hemacytometer (Hausser Scientific Horsham, PA). Conidial production was expressed as the mean number of conidia per surface area (m²) of V8 agar medium or per volume (milliliter) of liquid YPG medium. Experiments were carried out with three replicate Petri dishes or flasks.

Chemical analysis of cell wall

Conidia were inoculated into 40 ml of YPG liquid medium at a concentration of 10^6 conidia ml⁻¹ and germinated for 12 h at

28°C with shaking (80 r.p.m.). Then the Mycelium was harvested by filtering the culture through two layers of Miracloth (Calbiochem, CA) to eliminate conidia produced in liquid culture. Mycelium was then washed twice with distilled water and lyophilized. Three aliquots of 10–12 mg dry mycelium were used as independent samples for cell wall analysis. The same experiment was repeated twice. To remove unbound cell wall proteins and water soluble sugars, each sample was boiled in 2 ml of 2% SDS in 50 mM Tris-HCl buffer supplemented with 100 mM Na-EDTA, 40 mM β -mercaptoethanol and 1 mM PMSF (Elorza *et al.*, 1985) for 5 min (Schoffelmeyer *et al.*, 1999). Mannoprotein was extracted with 3% NaOH at 75°C for 1 h and quantitatively determined with Lowry protein assay (Lowry *et al.*, 1951). Glucan and chitin were digested in 96% formic acid at 100°C for 4 h. Formic acid was evaporated by lyophilizing and the residues were dissolved in 10 ml of distilled water. Glucan and chitin were estimated by determining the released glucose and N-acetylglucosamine after digestion. Glucose was measured by the phenol-sulphuric acid method (Dubois *et al.*, 1956). N-acetylglucosamine was measured by the method described by Lee *et al.* (2005).

mRNA studies

For Northern analysis of *HYD1-HYD5* genes and *FvVE1* gene, first mycelia from the wild-type strain M3120, and the corresponding *Fvve1* mutant and complementation strains were grown in 125 ml-flasks containing 40 ml of YPG medium for 14 h at 200 r.p.m. and 28°C. Then the mycelia from each flask was harvested by filtration using sterile Miracloth and transferred onto fresh solid YPG medium, where the cultures were incubated for an additional 72 h at 28°C. At that time, the mycelial samples were carefully scraped from the plates, frozen with liquid nitrogen and stored at –80°C. Total RNA was isolated from fungal mycelia using Triazol reagent (Invitrogen) according to the manufacturer's instructions. Approximately 20 μ g of total RNA for each sample was separated by agarose gel electrophoresis and blotted. Agarose gel purified DNA fragments representing the *F. verticillioides* hydrophibin genes *HYD1*, *HYD2*, *HYD3*, *HYD4*, *HYD5* and *FvVE1* coding regions were used to generate [α -³²P]dATP-labelled probes. Primers used to PCR-amplify *F. verticillioides* M-3120 genomic DNA to generate these probes were as follows: *HYD1F* 5'-GCAGTACATGACCATCGTCGC-3' and *HYD1R* 5'-CGTTTGCCTGGCATTCTTG-3', *HYD2F* 5'-GCCTACACTGTCATCGCTTTCC-3' and *HYD2R* 5'-CCTGGCAC TCCTTGTTGAGG-3', *HYD3F* 5'-AGTTCGCCAAGATC GCCT C-3' and *HYD3R* 5'-AGAGGGGCATCAGATTGGC-3', *HYD4F* 5'-GTTCTACGC CATCGCCTCTC-3' and *HYD4R* 5'-AACTGCACTCGCCGAAAG-3', *HYD5F* 5'-CAGTTC TCACTCGCTCTC GTG-3' and *HYD5R* 5'-TTTCCG CAGTCAAGGTCTGC-3' and *FvVE1F* 5'-CTTTGATCT GGAACCTGCCGATGGCGATCTTCCACCC-3' and *FvVE1R* 5'-GCATCCGTCTTGGAGTATGG-3'.

Transmission electron microscopy

Mycelia were fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer for 3 h. Mycelia were postfixed with 1% osmium tetroxide, dehydrated with a series of ethanol solu-

tions from 10% to 100% and embedded in EMBed-812 resin (Electron Microscopy Sciences, PA). Ultra thin sections made with a diamond knife (Diatome, PA) were stained with 2% uranyl acetate for 20 min followed by staining in 0.2% lead citrate for 40 min. A Hitachi H-600 transmission electron microscope was used. Images were recorded on Kodak Electron Microscope Film #4489; negatives were scanned using an Epson Perfection 2540 Photo scanner and Photoshop software.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Conidia from wild type (FGSC4), ΔveA and *FvVE1* complementation *A. nidulans* strains point-inoculated on YGT medium (Kato *et al.*, 2003) and incubated for 7 days. Close-up images were captured with a Spot Insight Color camera on a Leica MZ75 dissecting microscope with a 2.5 × objective. Bar, 1 mm.

Fig. S2. Observation of conidial types formed by *F. verticillioides* wild type, $\Delta Fvve1$ mutant and complementation strains.

A. Micrographs of microconidia from wild-type M3120 (left) and macroconidia from $\Delta Fvve1$ mutant M31206 (right) grown on V8 solid medium for 96 h at 28°C. Samples were fixed and stained with DAPI as described by Fernandez-Martinez *et al.* (2003). The presence of nuclei is indicated with arrows.

B. Micrographs of samples from the same origin stained with Calcofluor as described by Harris *et al.* (1994). Septa are indicated with arrows.

C. Conidia from the yeast-like growth observed in the $\Delta Fvve1$ mutants in YPG liquid culture incubated for 16 h at 200 r.p.m. and 28°C. Samples were stained with DAPI (left) or Calcofluor (right). Several tens of spores were observed for each strain before taking representative images. All images were captured using the same exposure with a light and epifluorescence NIKON600 microscope with a 100 × objective. Bar, 10 µm.

Fig. S3. Effect of the *Fvve1* mutation on sexual development in *F. verticillioides* strains.

A. Wild-type *MAT1-1* was fertilized with wild-type *MAT1-2* conidia.

B. Wild-type *MAT1-1* was fertilized with $\Delta Fvve1$ *MAT1-2* conidia.

C. $\Delta Fvve1$ *MAT1-1* was fertilized with wild-type *MAT1-2* conidia.

D. $\Delta Fvve1$ *MAT1-1* was fertilized with male $\Delta Fvve1$ *MAT1-2* conidia. A perithecius is indicated with an arrow. Images were captured on the 14th day after fertilization with a Spot Insight Color 3.2.0 camera attached to a Leica MZ75 dissecting microscope.

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